THERIMMUNE
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Pubertal Vinclozolin Study	RACB 20104
Pubertal Methoxychlor Study	RACB 20103
Pubertal Flutamide Study	RACB 20105
Pubertal Ethinyl Estradiol Study	RACB 20106

PUBERTAL TOXICITY STUDY OF VINCLOZOLIN AND FLUTAMIDE IN MALE SPRAGUE-DAWLEY RATS AND METHOXYCHLOR AND ETHINYL ESTRADIOL IN FEMALE SPRAGUE-DAWLEY RATS WHEN ADMINISTERED IN CORN OIL BY ORAL GAVAGE

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National Institute of Environmental Health Sciences (NIEHS)
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Testing Facility: TherImmune Research Corporation (TherImmune)
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1.0 INTRODUCTION

1.1 Proposed Investigations/Rationale for Dose Selection

Numerous pharmaceutical and environmental agents have been shown to alter the timing of pubertal development in mammals since puberty is a time of rapid interactive endocrine and morphological changes. The purpose of the current study is to provide data on proposed procedures to evaluate the effects of Vinclozolin, Methoxychlor, Flutamide and Estradiol on pubertal development in the intact juvenile/peripubertal male and female rat. The proposed design will detect agents that have antithyroid, estrogenic, androgenic, antiandrogenic activity, or alter puberty via changes in follicle stimulating hormone (FSH), luteinizing hormone (LH), prolactin, growth hormone (GH), or hypothalamic function (Stoker *et al*., 2000 and Goldman *et al*., 2000).

Vinclozolin, or 3-(3,5-dichlorophenyl)-5-methyl-5-vinylox-azolidine-2,4-dione, is a fungicide used on fruits, vegetables, turfgrass, and ornamental plants (U.S. EPA, 1998). In vivo, vinclozolin inhibits AR-dependent gene expression (Kelce et al., 1997) and produces a spectrum of anatomical defects. Administration of vinclozin (400 mg/kg) to rats on gestational day (GD) 14 through postnatal day (PND) 3 resulted in effects similar to those caused by flutamide, a well known AR antagonist. These effects include reduced anogenital distance (AGD); persistent nipples; cleft phalllus; hypospadias; reduces weights of the ventral prostate, seminal vesicles and epididymis; and reduces sperm count (Gray et al., 1994; 1999a). Exposing weanling male rats to the antiandrogenic pesticides p,p'- DDE or vinclozolin delays pubertal development in weanling male rats as indicated by delayed preputial separation and increases body weight at puberty. In contrast to delays associated with exposure to estrogenic substances, antiandrogens do not inhibit food consumption or retard growth (Anderson, *et al*., 1995b).

Flutamide (4'-nitro-3'-trifluoromethyl-isobutyranilide) is a potent nonsteroidal androgen receptor antagonist that has been used therapeutically to treat androgen-dependent prostrate cancer (Delaere and Van Thillo, 1991;Murphy *et al*., 1991) and as a tool to study male reproductive development. Studies in rats have demonstrated that pre- or postnatal flutamide (6.25 to 50 mg/kg) exposure alters androgen-dependent reporductive development(Imperato-McGinley *et al*., 1992; Kassim *et al*., 1997)and has been shown to produce decreased reproductive organ weights, feminization of male external genitalia, altered

androgen-dependent testicular descent, and retention of nipples when male offspring are exposed *in utero* (Imperato-McGinley *et al*., 1992).

Methoxychlor has been used for nearly 50 years for insect and larval control. Its advantages over DDT are that methoxychlor is more readily metabolized and excreted by mammalian systems (Kapoor *et al*., 1970), and therefore there is less bioconcentration than with DDT. This metabolism also yields mono- and bis-hydroxy metabolites of methoxychlor (Bulger et al., 1978), which helps explain both the uterotrophic effects noted earlier for methoxychlor (Tullner, 1961) and the observations that methoxychlor *in vivo* reduced the uterine uptake of radiolabeled estradiol (Welch *et al*., 1969). Treatment with methoxychlor at 5, 50, or 150 mg/kg for the week before and the week after birth to PND 7 resulted in unchanged anogenital distance, accelerated vaginal opening, delayed prepuce separation. Methoxychlor at 50 and 150 mg/kg disrupted adult estrous cyclicity and reduced epididymal sperm counts and testis weights

As cited by *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, (1996), estrogens are among the most commonly prescribed drugs in the United States. The two major uses are as components of combination oral contraceptives and hormone replacement therapy in postmenopausal women. The pharmacological considerations for estrogen use in oral contraceptives and post menopaousal hormone replacement are substantially different, primarily because of the doses used. Historically, conjugated estrogens have been the most common agents for postmenopausal use, and 0.625 mg/kg/day is effectivbe in most women. In contrast, most combination oral contraceptives in current use employ 20 to 35 ug/day of ethinyl estradiol. Conjugated estrogens and ethinyl estradiol differ widely in their oral potencies; for example, a dose of 0.625 mg of conjugated estrogens generally is considered equivalent to 5 to 10 ug of ethinyl estradiol.

Several authors have demonstrated the estrogenic responses to ethinyl estradiol in rodents. Laws *et al*. (2000) showed that *in vivo* studies indicated the 3-day uterotrophic assay in prepubertal rats was best for detecting estrogenic activity when compared with all other end points, based upon the dose-response data for ethinyl estradiol (0.01-0.1 mg/kg, oral), 4-tert-octylphenol (50-200 mg/kg, oral), and 4-nonylphenol (25-100 mg/kg, oral). Although oral doses of ethinyl estradiol (0.01 mg/kg) induced a significant increase in uterine weight in the prepubertal rat, this dose was ineffective for stimulating a

similar response in ovariectomized adult rats. The age of vaginal opening was advanced following oral exposure from postnatal days 21-35 to ethinyl estradiol (0.01 mg/kg). Ethinyl estradiol advanced the day of vaginal opening by 6.0 ± 0.18 days (30.6 days in control vs. 24.6 days in treated). In addition, the number of 4-5 day estrous cycles was reduced during a 25 day exposure to ethinyl estradiol (0.01 mg/kg).

Advanced vaginal opening was also demonstrated by Odum et al., (1997) using doses of 2-400 ug/kg/day, subcutaneous, and Singh and Kamgoj, (1980) using doses of 5 ug/kg/day for 5 days. Singh and Kamboj (1980) also showed an advance in cornified vaginal cells.

The data in this study should result in advanced vaginal opening, advanced first estrous and onset of estrous cycles, and/or persistent vaginal estrus in the ethinyl estradiol and methoxychlor females exposed *in utero* and in delayed preputial separation, decreased reproductive organ weights, altered external genitalia, and/or retention of nipples in the vinclozolin and flutamide males exposed *in utero.*

1.2 Regulatory Compliance

This study will be conducted according to a modification of the Health Effects Test Guidelines OPPTS 870.8300 Reproduction and Fertility Effects and in compliance with the Food and Drug Administration Good Laboratory Practice Regulations for Nonclinical Laboratory Studies (1987). All procedures will follow TherImmune Standard Operating Procedures.

1.3 Quality Assurance

The protocol, in-life phases, data, and the final report will be audited by TherImmune Quality Assurance. Critical phases to be audited for each generation will be selected by the Director of Quality Assurance.

1.4 Testing Facility

TherImmune Research Corporation (TherImmune)

15 Firstfield Road

Gaithersburg, MD 20878

2.0 TEST ARTICLE

2.1 Characterization of Test Articles

2.1.1 Vinclozolin (from MSDS)

Identity: Vinclozolin

R.O.W. ID No.: 1317B

Source: Battelle Organic Synthesis Group

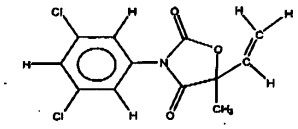
CAS No.: 50471-44-8

Lot No.: 102996

Molecular Wt: 286.1g/mol

Formula: $C_{12}H_9NO_3CL_2$

Structure:



Purity: 99.6%

Storage:

Test Article: Store at room temperature (~25°C) and protected from light.

Formulation: Stored in sealed amber glass bottles at room temperature (~25°C) and protected from light

Stability:

Test Article: Analyze every 24± 2 weeks to verify stability.

Formulation: Dose formulations (2 mg/mL) are stable for 42 days at temperatures of 25°C, 5°C, and -20°C in sealed amber glass bottles and protected from light.

2.1.2 Methoxychlor (from MSDS)

Identity: Methoxychlor

R.O.W. ID No.: 1321B

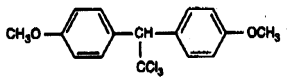
Source: Sigma Chemical Co.

CAS No.: 72-43-5

Lot No.: 124F0575

Molecular Wt: 345.7

Formula: $C_{16}H_{15}Cl_3O_2$

Structure: 

Purity: 95%

Storage:

Test Article: Stored in a sealed container under nitrogen and protected from light at ambient temperature (23 to 28°C)

Formulation: Stored in sealed glass vials at refrigerator temperature.

Stability:

Test Article: Analyze every 24± 2 weeks to verify stability.

Formulation: Dose formulations (1.82 mg/mL in corn oil) are stable for 30 days under refrigerated conditions (2 to 5°C) conditions for 23 days under ambient (23 to 28°C) conditions. Under conditions which simulate animal dosing (room temperature, exposed to air in hood), the dosage formulation showed no appreciable loss.

2.1.3 Flutamide:

Identity: Flutamide

R.O.W. ID No.: 1198E

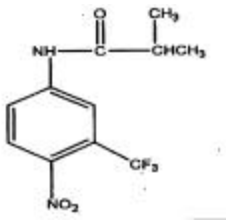
Source: Sigma Chemical Co.

CAS No.: 13311-84-7

Lot No.: 109H0952

Molecular Wt: 276.2 g/mol

Formula: $C_{11}H_{11}F_3N_2O_3$

Structure: 

Purity: 99%

Storage:

Test Article: Store at room temperature (~25°C) in sealed amber glass bottles.

Formulation: Store in sealed amber glass bottles at 5°C or -20°C, protected from light.

Stability:

Test Article: Analyze every 24± 2 weeks to verify stability.

Formulation: Dose formulations (10 mg/mL in corn oil) are stable for 42 days at 5°C or -20°C, with -20°C being preferable. Under conditions which simulate animal dosing (room temperature, exposed to air in hood), the dosage formulation showed no appreciable loss.

2.1.4 Ethinyl Estradiol

Identity: Ethinyl estradiol

R.O.W. ID No.: 1318B

Source: Sigma Chemical Co.

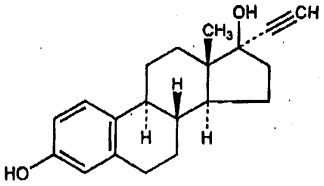
CAS No.: 57-63-6

Lot No.: 45H0716

Molecular Wt: 296.44

Formula:

Structure:



Purity: 99.7%

Storage:

Test Article: Store in sealed amber glass bottles away from light at ambient temperatures (23 to 28°C) under inert headspace.

Formulation: Store in sealed amber glass bottles away from light and refrigerated (~5°C).

Stability:

Test Article: Analyze every 24± 2 weeks to verify stability.

Formulation: Dose formulations (1.0 µg/mL in corn oil) are stable for up to 14 days under refrigerated conditions (~5°C). Under conditions which simulate animal dosing (room temperature, exposed to air and light), the dosage formulation showed no appreciable loss.

2.1.5 Certificate of Analysis

Each batch of each test article will be accompanied by a certificate of analysis. The Sponsor will determine for each batch of test article the strength, purity, and composition or other characteristics that appropriately define the test article. A copy of the dose formulation report will be attached (Appendix 2).

2.1.6 Bulk Chemical Samples

Prior to use, two 5 g samples of the bulk of each test article will be collected into a glass bottle with Teflon® coated lids, sealed and stored in the freezer (-20EC) protected from light for possible future reanalysis.

A bulk test article sample of 5 grams will be collected and sent to a NTP subcontractor for purity and stability testing within 30 days of receipt, and thereafter at 24±2 week intervals. A 35 ml aliquot will be sent within 30 days prior to the start of any study.

2.2 Safety and Handling

The precautions necessary when handling any test article or the prepared formulations of the test substance are based on the Material Safety Data Sheet (MSDS) supplied by the Sponsor. The MSDS will be retained in the study file.

2.2.1 Emergency First Aid Procedures

Eye: First check the victim for contact lenses and remove if present. Flush victims eyes with water or normal saline solution for 20 to 30 minutes while simultaneously calling a hospital or poison control center. Assure adequate flushing. Do not put any ointments, oils, or medication in the victims eyes without specific instructions from a physician. Immediately transport the victim to a hospital even if no symptoms (such as redness or irritation) develop.

Skin: IMMEDIATELY flood affected skin with water while removing and isolating all contaminated clothing. Gently wash affected skin areas thoroughly with soap and water. If symptoms such as inflammation or irritation develop, IMMEDIATELY call a physician or go to a hospital for treatment.

Inhalation: IMMEDIATELY leave the contaminated area and take deep breaths of fresh air. If symptoms (such as wheezing, coughing, shortness of breath, or burning in the mouth, throat, or chest) develop, call a physician and be prepared to transport the victim to a hospital.

Ingestion: If the victim is conscious and not convulsing, give 1 or 2 glasses of water to dilute the chemical and IMMEDIATELY call a hospital or poison control center. If the victim is

convulsing or unconscious, ensure that the victim's airway is open and lay the victim on his/her side with the head lower than the body. DO NOT INDUCE VOMITING. IMMEDIATELY TRANSPORT THE VICTIM TO A HOSPITAL.

2.2.2 Protective Equipment

Eye:	Safety glasses/goggles
Gloves:	Two pairs of dissimilar protective gloves shall be worn when handling the neat chemical, and dosing solutions.
Clothing:	Minimally, a disposable laboratory suit (e.g. Tyvek ®), bouffant, and shoe covers shall be worn, as specified in the most current NTP Statement of Work or the NTP Health and Safety Minimum Requirements.
Respiratory Protection:	A NIOSH-approved chemical cartridge respirator with an organic vapor, acid gas and high-efficiency particulate filter cartridge. Use only in well ventilated areas.

2.2.3 Spills and Containment

The Health and Safety Officer shall be informed in the event of any spillage. If the spillage is containable (at the discretion of the Health and Safety Officer) the following steps shall be taken:

1. A HAZORB® Chemical Spill Kit will be used.
2. Place HAZORB® control pillows around the spill area.
3. Place additional pillow over spill and allow absorption to occur.
4. Dispose of all absorbed material as hazardous waste.

If the spillage is not containable (at the discretion of the Health and Safety Officer), self contained breathing apparatus will be used.

2.2.4 Decontamination of Laboratory Equipment

Computer Terminal/ General Equipment	Whenever feasible, a protective covering (*e.g.*, plastic wrap) shall be placed over the keyboard when in use. Before removing general laboratory equipment (*i.e.*, lab carts, portable hoods and balances) from animal dosing rooms and/or chemical preparation areas, clean work surfaces with a 1% T.B.Q (quaternary ammonium) solution.

2.2.5 Disposal Procedures

Waste Disposal:	Securely package and label, in double bags, all waste material. All potentially contaminated material (*i.e.*, carcasses, bedding, soiled disposable clothing) shall be disposed of by incineration in a chemical incinerator equipped with an after burner and scrubber in a manner consistent with federal (EPA), state, and local regulations.

2.3 Dose Formulation and Analysis

The quantity of Vinclozolin and Methoxychlor, Ethinyl estradiol, and Flutamide to prepare a solution will be accurately weighed into a volumetric flask. The vehicle (corn oil) will be added to the required volume and the solution stirred for at least 10 minutes to insure complete dissolution. The formulation for each group will be dispensed into daily aliquots which will be stored in glass bottles with Teflon® coated lids protected from light at 2-9EC. Each solution will be stirred prior to dosing.

Each formulation will be labeled with the TherImmune No., R.O.W. ID No., Group, Dose Level, Vehicle, Mix Number, Preparation Date, Storage conditions, Study and color coded by group. The tray used to hold the daily aliquots will be labeled with the TherImmune Study No., Task No., R.O.W. ID No., Test article Name, Group, Dose Level, Vehicle, Mix Number, Preparation Date, Expiration Date, Storage conditions, Study and Group Color Code (see SOP No 506.0 Storage Sampling, and Labeling of Control and Test Diets and Mixtures and SOP No.121.0 Color-Coding for Study Identification and Dose Groups).

Every time a new mix or batch of chemical is prepared three (3) 35 ml archival samples of each dose level of test article formulation will be collected and stored at TherImmune in glass bottles with Teflon® coated lids protected from light in the refrigerator. One set of samples from each dose level will be forwarded on ice packs to NTP analytical chemistry subcontractor for dose concentration analysis at the following times: initial, mid, and final formulations and at other periods specified by the Sponsor and communicated to the Study Director. If the formulation are suspensions, three archival samples (35 ml) will be collected from the top, mid, and bottom of the low and high dose formulations in glass bottles with Teflon® coated lids. A 5 g sample of bulk test article will be forward to the NTP analytical chemistry subcontractor for stability analysis prior the initial mix and thereafter at 24 ± 2 week intervals. Archival samples which are not selected for analysis will be discarded as hazardous waste as following requirements in Section 2.2.6 after at least ninety days following preparation.

3.0 EXPERIMENTAL DESIGN

3.1 Test System

Species:	Sprague-Dawley Crl: CD® (SD) IGS BR
Rationale:	The Sprague-Dawley rat was selected as the test system due to its established quality as a breeder and the availability of historical toxicologic data for reference.
Supplier:	Charles River Breeding Laboratories. (Portage, Michigan or Raleigh North Carolina)
Number/Sex:	Thirty time-mated females plus 5 extras will arrive on gestation day (GD) 7-10 (day of mating = GD 0)
Age (at study initiation):	Approximately 12 weeks

3.2 Animal Husbandry

All laboratory animal care will be in accordance with the Guide for the Care and Use of Laboratory Animals, TherImmune Standard Operating Procedures, and applicable FDA regulations.

Acclimation period: At least 7 days

Animal housing during acclimation:	1 /cage
Lighting:	14/10 hour light/dark cycle
Temperature:	22±2°C
Relative Humidity:	40-50%
Observations:	Twice daily observations for general health and availability of adequate food and water.
Cage changes:	At least twice a week, unless the animals are individually housed in 19" x 10½" x 8" (group-housed) cages which may be changed once a week.
Feeder/bottle changes:	At least once per week
Procedure for Individual Animal Identification:	All animals will be uniquely identified by tail tattoo and by cage cards.
Housing Requirements:	
Cage Type:	Polycarbonate
Cage Measurement:	19" x 10 1/2" x 8" (group housed) 9" x 8 1/2" x 8" (single housed) (20x25x47cm)
Bedding Material:	"Sani Chip" Hardwood Laboratory Bedding (P.J. Murphy Forest Products Corp., Montville, N.J.). All bedding will be autoclaved prior to use.
Feed:	Teklad Certified Rodent Diet 7012C
Frequency:	*ad libitum*
Analysis:	The feed is analyzed for nutrients, aflatoxins, nitrosamines, heavy metals, chlorinated hydrocarbons, organophosphates, PCB's, nitrates, nitrites, BHA, BHT, total bacterial plates, coliforms, E. coli and Salmonella by the vendor prior to release.
Water:	Filtered tap water
Frequency:	*ad libitum*
Analysis	A water quality sample is analyzed for total dissolved solids, heavy metals, chlorinated hydrocarbons, organophosphates, nitrates, nitrites, microbiological content, and total trihalomethanes at least semi-annually to conform with the Safe Drinking Water Act. None of the contaminants are expected to be at levels sufficient to interfere with the study.
Health Screening Requirements:	Prior to initiation of the study one female will be sent to AnMed/Biosafe Laboratories (Rockville, MD) for serological tests: Pneumonia Virus of Mice (PVM) Respiratory Enteric Orphan III (REO3) Toolan's H-1 (parvovirus) (TH1)

Encephalomyelitis (GD7)
Sialodacryoadenitis Virus (coronavirus)(SDAV/RCV)
Sendai (SEN)
Mycoplasma Pulmonis (MYCO)
Lymphocytic Choriomeningitis (LCM)
Kilham's rat Virus/Rat Orphan Parvovirus (KRV/rOPV)

4.0 STUDY DESIGN

4.1 General Study Design

Thirty time-mated females will be used on study and will produce juvenile animals. Ninety juvenile males will be assigned on a male cohort study and ninety juvenile females will be assigned on a female cohort study.

Definition: Gestation Day 0 (GD 0) = Day of mating (Sperm +)
Postnatal Day 0 (PND 0) = Day of Delivery

4.1.1 Mortality

Any animals found dead or killed *in extremis* on the study will be subject to necropsy. The following tissues will be retained and placed in Bouin's then transferred into 70% ethanol within 24-48 hours:

liver
kidneys
thymus
adrenals
spleen
both testes and epididymis (male)
prostate (ventral and dorso-lateral lobes) (male)
seminal vesicles/coagulating glands (male)
vagina/uterus/cervix (female)
ovaries (female)
gross lesions
pituitary
brain
stomach
thyroid/parathyroids

Histopathology of the unscheduled deaths/sacrifices will be performed at the discretion of the Sponsor.

4.1.2 Necropsy

All necropsies are performed according to TherImmune Standard Operating Procedures.

4.1.3 Histopathology

Histopathological examination of fixed tissues for animals found dead or killed *in extremis* will not be conducted unless indicated by a protocol amendment. Tissues will be transferred to Pathology Associates International (PAI) located in Frederick, MD under subcontract to TherImmune. If pathology is conducted, the findings will be incorporated in the final report.

4.2 Juvenile Animal Production

All dams will be housed individually from the day of receiving to the day of euthanasia. Thirty females will be allowed to deliver litters to be used on study. Litters will be culled up to 8-10 pups on PND 4 and approximately equal numbers of male and female pups will be kept in each litter. On PND 21, all male and female pups will be separated from dams and ninety males will be assigned to the juvenile male

study and ninety females will be assigned to the juvenile female study. Selection will be made by weighing all pups and selecting the 90 animals of each sex that are most similar in weight. All extra animals will be removed from study and discarded without necropsy on the day of separation.

4.2.3 Allocation

Thirty time-mated females plus 5 extras will be ordered from Charles River and arrived on gestation day 7-10 (day of mating = gestation day 0).

4.2.4 Treatment

There is no treatment for time-mated females.

4.2.5 Measurements

Dams

Observations for mortality and clinical signs:	Twice daily
Body Weight:	At littering At PND 21

Pup Observations

The following pup observations will be made for the F_1 pups

PND 0: Number of live pups
Number of dead pups
Number of males
Total body weight of males
Number of females
Total body weight of females

PND 4: Litters will be culled to 8-10 pups (approximately equal numbers of male and female pups, if possible). Pups not selected will be discarded without necropsy.

Body weights: Weekly
Individual pup weights will be collected on PND 21 and recorded to the nearest 0.1 gram.

4.2.6 Disposition of Offspring and Dams

Dams: Discarded without necropsy on PND 21 following the terminal body weight collection.

Pups: Animals that are not selected for juvenile male or female cohort studies will be discarded without necropsy on PND 21.

4.3 Juvenile Male Cohort Study:

4.3.1 Number of Animals and dose levels:

Group No.	Test Material	Dose Level (mg/kg/day)	Number of Males

1	Corn Oil Vehicle	0	15
2	Vinclozolin	10	15
3	Vinclozolin	30	15
4	Vinclozolin	100	15
5	Flutamide	25	15
6	Flutamide	50	15

4.3.2 Allocation

After PND 21 body weight collection, the male pups in each litter will be assigned to treatment groups in a randomized block-fashion based upon weight, with 6 males per block. The 15 blocks range from heaviest to lightest. Each treatment group then gets one pup from each of the 15 blocks.

4.3.3 Treatment

Starting on PND 23, animals will be administered by oral gavage at 2.5 ml/kg/day (using 18gauge gavage needle at 1 inch length with a 2.25 mm ball and a 1 cc glass disposable tuberculin syringe) once daily at 0700-1000 each day and continue through necropsy. Control animals will receive the vehicle, corn oil, only. The formulations will be stirred before and while dosing. Dose volumes will be calculated daily based on same day body weight.

On the day of termination, animals will be dosed between 0700 to 0900.

4.3.4 Measurements

Observations for mortality and signs of toxicity:	Twice daily
Body Weight:	PND 23 and daily thereafter, including preputial separation and termination
Physical Examination:	PND 23 and weekly thereafter At termination
Preputial Separation Observation:	Preputial separation will be observed on all males daily starting on PND 23. A partial separation, complete separation, and persistent thread of tissue between the gland and prepuce will be recorded. However, the day of complete separation is the endpoint used in the analysis for the age of preputial separation.

4.3.5 Termination

Schedule:	On PND 53-54
Groups:	The necropsy will examine all treated males from each dose group.
Procedures:	Care must be taken to remove mesenteric fat from the sex accessory glands with small scissors such that the fluids are retained. Once free from the fat and adnexa, the weight with fluid is recorded. The seminal vesicle with coagulating gland is then placed on a paper towel, pressed so that the fluid is exuded, gently blotted dry, and reweighed.

Small tissues such as the adrenals and pituitary, as well as tissues that contain fluid, will be weighed immediately to prevent partial drying prior to weighing.

Organ Weights: The following organs will be weighed and recorded to the nearest 0.1 mg.
adrenal glands (paired)
epididymis (right and paired weights)
kidneys (paired)
levator ani plus bulbocavernosus
liver
pituitary
seminal vesicle and coagulating gland with and without fluid
testes (paired)
thyroid/parathyroids
ventral and dorsal-lateral prostate, separately

Tissue Preservation:
After weighing, the following tissues will be placed in Bouin's and transferred into 70% ethanol within 24-48 hours:
adrenal glands (paired)
epididymides
kidneys (paired)
liver
pituitary
testes
thyroid/parathyroids (with attached portion of the trachea)
gross lesions

Histopathology: The thyroid, testes, and epididymides from all males/group will be embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically. All gross lesions from all males/group will be embedded in paraffin, sectioned, and examined microscopically by the study pathologist.

4.4 Juvenile Female Cohort:

4.4.1 Number of Animals and dose levels

Group No.	Test Material	Dose Level (mg/kg/day)	Number of Females
1	Corn Oil Vehicle	0	15
2	Methoxychlor	12.5	15
3	Methoxychlor	25	15
4	Methoxychlor	50	15
5	Ethinyl Estradiol	0.0025	15
6	Ethinyl Estradiol	0.005	15

4.4.2 Allocation

After PND 21 body weight collection, the female pups in each litter will be assigned to treatment groups in a randomized-block-fashion based upon weight with 6 females per block. The 15 blocks range from heaviest to lightest. Each treatment group then gets one pup from each of the 15 blocks.

4.4.3 Treatment

Starting on PND 22, animals will be administered by oral gavage at 2.5 ml/kg/day day (using 18gauge gavage needle at 1 inch length with a 2.25 mm ball and a 1 cc glass disposable tuberculin syringe), once daily at 0700-1000 each day, and continue through necropsy. Control animals will receive the vehicle, corn oil, only. The formulations will be stirred before and while dosing. Dose volumes will be calculated daily based on same day body weight.

On the day of termination, animals need to be dosed during 0700 to 0900.

4.4.4 Measurements

Observations for mortality and signs of toxicity:	Twice daily
Body Weight:	PND 22 and daily thereafter
Physical Examination:	PND 22 and weekly thereafter At termination
Vaginal Opening Observation:	Vaginal opening will be observed on all females daily starting on PND 22. The appearance of a small pin hole, a vaginal thread, and complete vaginal opening are recorded. However, the day of complete vaginal opening is the endpoint used in the analysis for the age of vaginal opening. If vaginal opening does not occur by PND 42 then PND 43 may be used to determine the mean for the age at vaginal opening. In this case, the number of females that did not reach vaginal opening by necropsy within each treatment group should also be included in the data summary.

Estrous Cyclicity: Beginning on the day of vaginal opening and continuing through day of necropsy, daily vaginal smears are obtained and evaluated under a low-power light microscope for the presence of leukocytes, nucleated epithelial cells, or cornified epithelial cells to determine the age of the first vaginal cycle and/or any effects on estrous cyclicity. Extended estrus shall be defined as exhibiting cornified cells with no leukocytes for 3 or more days and extended diestrus as the presence of leukocytes for 4 or more days.

4.4.4 Termination

Schedule: On PND 42-43

Groups: The necropsy will examine all treated females from each dose group.

Procedures: Care must be taken to remove mesenteric fat from the uterine horns and to avoid damaging the uterus so that the uterine fluid is retained. The uterus plus cervix are separated from the vagina and the weight of the uterus with fluid is recorded. The uterus is then placed on a paper towel, slit to allow the fluid contents to leak out, gently blotted dry and reweighed.

Small tissues such as the adrenals and pituitary, as well as tissues that contain fluid, will be weighed immediately to prevent partial drying prior to weighing.

Organ Weights: The following tissues will be weighed and recorded to the nearest 0.1 mg.
adrenal glands (paired)
kidneys (paired)
liver
ovaries (paired)
pituitary
thyroid/parathyroids
uterus and cervix with and without fluid

Tissue Preservation:
After weighing, the following tissues will be placed in Bouin's and transferred into 70% ethanol within 24-48 hours:
adrenal glands (paired)
kidneys (paired)
liver
ovaries (paired)
pituitary
thyroid/parathyroids (attached with the portion of the trachea)
uterus and cervix
gross lesions

Histopathology: The ovaries, uterus and cervix, and gross lesions from all females/group will be embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically by the study pathologist.

5.0 PROPOSED STATISTICAL ANALYSES

All raw data will be sent in Excel spreadsheet (soft copy) to the Project Officer at the same time that the data are sent to the statistical support group.

Statistical analyses of the following will be performed:

Data from the main study will be analyzed by a statistical support group under contract to NTP/NIEHS, RTP, NC. A statistical analysis report will be submitted to TherImmune by the contractor for inclusion in the final study report. All data (age and weight at vaginal opening/preputial separation, body and organ weights at necropsy) are analyzed using ANOVA. Organ weights may be analyzed by ANCOVA using the body weight at necropsy as a covariate. When significant treatment effects are observed, treatment means are tested using an appropriate multiple comparison test. Data should be evaluated for heterogeneity of variance by an appropriate statistical test and if present, data should be transformed or analyzed using a suitable non-parametric test.

6.0 REPORTS

The following reports will be submitted:

Draft Study Report

Thirty days after completion of all analysis, all data will be summarized and conclusions on the reproductive toxicity of the test article will be submitted to the Sponsor.

An executive summary will be prepared describing the number and strain of rats observed, the doses used for each chemical tested, and the effects and level of statistical significance for all required endpoints specified in the protocol for the maternal cohort, neonates, uterotrophic cohort and pubertal female/male cohorts. Raw data from each individual animal should be presented in a spreadsheet format. Data summary tables containing the mean, standard error of the mean (SEM), and sample size for each treatment group should be provided for all endpoints. The mean, SEM and CV values for the control data are examined to determine whether they meet acceptable QA criteria for consistency with historical values. Organ weight data may also be presented after covariance adjustment for body weight, but this should not replace presentation of the unadjusted data. In addition, the data tables are accompanied by summary of histological findings with photomicrographs of significant observations.

Final Study Report

The Final Study Report will be submitted to the Sponsor after the submission of the Draft Study Report.

7.0 STORAGE OF RECORDS

Upon submission of the final report, all original study records, including all original data sheets; all computer generated data; the original final report; tissues, computer printouts generated in the statistical analysis of data; and copies of the final report will be forwarded to the contracting agency, the NIEHS, Research Triangle Park, North Carolina. Copies of the final study report will also be filed with TherImmune.

8.0 PERSONNEL

Project Officer: Jack Bishop, Ph.D. (NTP)

Study Director:	Gary W. Wolfe, Ph.D., D.A.B.T
Reproductive Toxicologist:	Larissa B Nehrebeckyj, B.S.
Technical Supervisor:	Roland Naawu, M.S., LATG
Health and Safety Officer/ Facility Manager:	Robert Blackford, A.A., LATG
Veterinarian:	Edward Greenstein, D.V.M, ACLAM
Quality Assurance Officer:	Jim Carignan, B.S.
Report Manager:	Rita Patel, B.S.
Dose Preparation Supervisor:	Gary Holley, B.S.

9.0 SUBCONTRACTORS

Necropsy/Pathology:	PAI, Frederick, MD
Serology	AnMed/Biosafe, Rockville, MD
Clinical Chemistry	AniLytics, Inc., Gaithersburg, MD

10.0 REFERENCES

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PUBERTAL TOXICITY STUDY OF VINCLOZOLIN AND FLUTAMIDE IN MALE SPRAGUE-DAWLEY RATS AND METHOXYCHLOR AND ETHINYL ESTRADIOL IN FEMALE SPRAGUE-DAWLEY RATS WHEN ADMINISTERED IN CORN OIL BY ORAL GAVAGE

Appendix 1: SCHEDULE

TO BE ADDED

PUBERTAL TOXICITY STUDY OF VINCLOZOLIN AND FLUTAMIDE IN MALE SPRAGUE-DAWLEY RATS AND METHOXYCHLOR AND ETHINYL ESTRADIOL IN FEMALE SPRAGUE-DAWLEY RATS WHEN ADMINISTERED IN CORN OIL BY ORAL GAVAGE

Appendix 2: DOSE FORMULATION REPORTS IN STUDY DATA

VINCLOZOLIN
METHOXYCHLOR
FLUTAMIDE
ETHINYL ESTRADIOL

PUBERTAL TOXICITY STUDY OF VINCLOZOLIN AND FLUTAMIDE IN MALE SPRAGUE-DAWLEY RATS AND METHOXYCHLOR AND ETHINYL ESTRADIOL IN FEMALE SPRAGUE-DAWLEY RATS WHEN ADMINISTERED IN CORN OIL BY ORAL GAVAGE

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	Personal Protective Equipment	1003.0, CHP, RPP
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	Spill Clean-Up, Accident and Emergency	1007.0, CHP
	Response (including material disasters) and	
	fires/explosions	
	Dose Preparation	500 Series
	Enter and Exit from Limited Access Areas	117.0, CHP, RPP
	Respiratory Protection and Fit	RPP

Note: CHP = Chemical Hygiene Plan
RPP = Respiratory Protection Program

STANDARD OPERATING PROCEDURES FOR HEALTH AND SAFETY

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